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SELECTIVE AND REVERSIBLE INHIBITION OF THE ATPase OF *MICROCOCCUS DENITRIFICANS* BY 7-CHLORO-4-NITROBENZO-2-OXA-1,3 DIAZOLE

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Summary

The covalent inhibitor of the beef heart mitochondrial ATPase 7-chloro-4-nitrobenzo-2-oxa-1,3 diazole inhibits the ATPase of phosphorylating particles prepared from *Micrococcus denitrificans*. Inhibition of both ATP synthesis and ATP hydrolysis occurs at similar rates, with a similar pH dependence, and in each case the inhibition is relieved by treatment with dithiothreitol. These results are compared with those previously obtained with the mitochondrial ATPase.

The beef heart mitochondrial ATPase can be inhibited by the chemical modifying reagent 7-chloro-4-nitrobenzo-2-oxa-1,3 diazole (NBD-Cl) [1]. The enzyme is inhibited both in the soluble form (coupling factor F_1) and in submitochondrial particles. Treatment with dithiothreitol restores activity in each case by removing the NBD group from the ATPase. It has been suggested previously that the inhibition is a result of selective modification of an essential tyrosine residue in the protein [1,2]. We have examined the effect of NBD-Cl on phosphorylating particles prepared from *M. denitrificans* to determine whether a similar inhibition occurs with a bacterial ATPase. These particles show respiratory control [3,4] and it was therefore possible to assay for oxidative phosphorylation by measuring the increased respiratory rate observed on addition of ADP [5].

Abbreviation: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3 diazole.

Incubation of the *M. denitrificans* particles with NBD-Cl abolishes the ADP-induced respiratory stimulation, without significantly affecting the respiratory rate observed in the absence of ADP. Only a slight inhibition of uncoupler-stimulated respiration is observed under these conditions (Fig. 1, Table I). Inclusion of dithiothreitol in the assay medium essentially restores the ADP-induced respiratory response to that observed in the absence of NBD-Cl treatment (Table I). Glutathione is as effective as dithiothreitol. The ATPase activity of these particles is also inhibited by NBD-Cl, and restored by the addition of either dithiothreitol or glutathione (Table I).

TABLE I

Inhibition of oxidative phosphorylation and ATPase activity in particles of *M. denitrificans* by NBD-Cl and reversal by dithiothreitol. The particles were incubated with NBD-Cl at pH 8.0 for 20 min at 20 °C. Respiration rates were determined as described in Fig. 1, except that, as indicated, 0.2 mM dithiothreitol was added to the assay medium 1—2 min before the addition of NAD^+. ATPase activity was measured by monitoring phosphate release in an assay medium containing 5 mM ATP, 5 mM $MgCl_2$, 20 mM Tris—acetate, 150 mM sodium bicarbonate pH 8.0.

Additions		Respiration Rate (μg atoms $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein)			ATPase activity (μmoles $P_i \cdot min^{-1} \cdot mg^{-1}$ protein)
Incubation Medium	Assay Medium	—	+ ADP	+ Uncoupler	
—	—	0.51	1.07	3.08	0.15
—	Dithiothreitol	0.53	1.14	2.92	0.17
NBD-Cl	—	0.51	0.51	2.23	0.02
NBD-Cl	Dithiothreitol	0.51	0.97	2.60	0.17
—	—	—	—	—	0.03*

* Omission of the sodium bicarbonate resulted in the decreased ATPase activity shown.

The pH at which NBD-Cl is incubated with *M. denitrificans* particles significantly influences the rate at which both phosphorylation and ATPase activity are lost (Fig. 2). The time courses for the inhibition of both processes at each pH tested are very similar (Fig. 2).

The ATPase activity was measured in a medium containing 150 mM sodium bicarbonate as this was found to stimulate ATPase activity by up to ten-fold. The reason for this bicarbonate stimulation is not yet clear. After NBD-Cl treatment there remained a small residual ATPase activity which was not stimulated by sodium bicarbonate (Fig. 2, Table I). This is perhaps the second type of ATPase activity proposed in earlier work on *M. denitrificans* [6].

Whereas the modes of action of most inhibitors used in studies of oxidative phosphorylation are as yet unknown, the action of NBD-Cl can be understood in chemical terms. Under the conditions of our experiments sulphydryl groups of cysteine and the phenolic oxygen groups of tyrosine can be modified covalently by NBD-Cl [1,2,7]. The different absorption spectra of the cysteine and tyrosine NBD derivatives allow a distinction to be made between the two possible reactions of NBD-Cl [1,7]. However, it has not

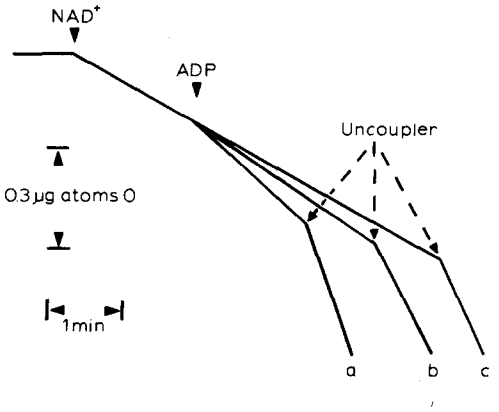

Fig.1. Effect of NBD-Cl respiratory control in phosphorylating particles of *M. denitrificans*. Particles, prepared essentially as described previously [3,4] were incubated at a concentration of 1.8 mg protein per ml in a medium containing 50 mM Tris—acetate, pH 7.5, and 0.2 mM NBD-Cl (from Serva Feinbiochemica) at 20 °C for (a) 0 min, (b) 10 min and (c) 30 min. Samples (0.1 ml) were taken for assay in a medium containing, in a total volume of 3 ml: 10 mM Tris—phosphate, pH 7.3, 5 mM magnesium acetate, 0.2 mg yeast alcohol dehydrogenase (from Sigma), and 30 µl ethanol. The reaction was started by the addition of NAD^+ (0.6 mM), subsequently ADP (0.2 mM) and, as uncoupler, [5] gramicidin D (1 µg) plus ammonium acetate (30 mM) were added as indicated. Oxygen uptake was measured at 30 °C with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England).

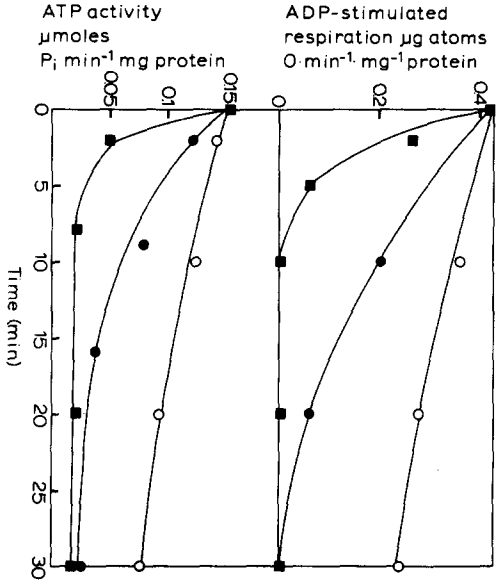

Fig.2. The effect of the pH at which particles of *M. denitrificans* are incubated with 0.2 mM NBD-Cl on the rate at which oxidative phosphorylation and ATPase activity are inhibited. ○, pH 7.0, ●, pH 7.5, ■, pH 8.0. Activities were assayed as described in the legends to Fig. 1 and Table I. Incubations with NBD-Cl were at 20 °C.

been possible to follow spectrophotometrically the reaction of NBD-Cl with the particles of *M. denitrificans*, mainly because of the relatively high absorption of the particles at the appropriate wavelength. However, several observations indicate that the modifications of the ATPases of *M. denitrificans* and mitochondria are related. The rates of inactivation of mitochondrial and *M. denitrificans* ATPases by NBD-Cl are similar. In addition the pH dependence of the rate of inhibition of the bacterial ATPase closely resembles that of the mitochondrial ATPase (Ferguson, S.J., Lloyd, W.J. and Radda, G.K., unpublished). Furthermore, no inhibition of the bacterial ATPase was observed on treating the particles with either 10 mM *N*-ethyl maleimide or 10 mM iodoacetamide in 50 mM Tris—acetate at pH 7.5 for up to 10 h. Under such conditions these reagents will react with sulphydryl groups of cysteine residues in proteins. Therefore, as the ATPase complex is not susceptible to either of these reagents, it is likely that NBD-Cl is modifying an essential tyrosine residue of the bacterial ATPase rather than a cysteine sulphydryl group, though it is difficult to exclude the possibility that NBD-Cl gains access to a sulphydryl group which is inaccessible to *N*-ethyl maleimide and iodoacetamide. However, the sulphydryl group of glutathione is not expected to be efficient at removing the NBD group from a buried amino acid residue. Therefore the finding that glutathione is as effective as dithiothreitol in restoring activity to the ATPase, suggests that NBD-Cl does not react with a buried residue of the *M. denitrificans*—ATPase complex.

The results presented in this report are consistent with the view that inhibition of ATP synthesis and hydrolysis is the result of a modfication of the ATPase complex by NBD-Cl. The similarity of the inhibition of the bacterial ATPase to the inhibition observed in submitochondrial particles suggests that it is a common feature of the two types of ATPase which is being modified. Further support for this proposal may come from studies of the isolated ATPase from *M. denitrificans* when the reaction with NBD-Cl can be studied spectrophotometrically. Our data are also relevant to the question of whether or not there are separate sites for ATP synthesis and hydrolysis [8]. In our view, a common site for both processes is indicated by the data with NBD-Cl, since it is unlikely that there could be a similar rate of chemical modification at different sites on the ATPase complex. Finally, it may be that NBD-Cl will be a general inhibitor of ATPases involved in oxidative phosphorylation.

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